Enterococci populations of a metropolitan river after an extreme flood event: prevalence, persistence and virulence determinants

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ABSTRACT

We investigated the prevalence, persistence and virulence determinants of enterococci populations in water samples collected over three rounds following an extreme flood event in a metropolitan river. Enterococci ($n = 482$) were typed using the high resolution biochemical fingerprinting method (PhP typing) and grouped into common (C) or single (S) biochemical phenotypes (BPTs). In all, 23 C-BPTs (72.6% of isolates) were found across the sites. A representative isolate of each C-BPT was identified to the species level and tested for the presence of seven virulence genes (VGs), biofilm formation and resistance to 14 antibiotics. The enterococci concentrations in samples collected during the first two rounds were above national recreational water guidelines. By round three, enterococci concentrations decreased significantly ($P < 0.05$). However, 11 C-BPTs (55.5% of isolates) persisted across all sampling rounds. *E. casseliflavus* and *E. mundtii* were the most common enterococci populations comprising of >57% of all isolates. Ten of the 11 most dominant C-BPTs were resistant to multiple antibiotics and harboured one or more VGs. The high prevalence of antibiotic resistance and VGs among enterococci isolates in this catchment not only provides them with niche advantages but also poses a risk to public health.

Key words | *Enterococcus* spp., extreme flood, population dynamics, virulence determinants

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INTRODUCTION

Enterococci inhabit the gastrointestinal (GI) tract of many animals, including humans (Abriouel *et al.* 2008; Byappanahalli *et al.* 2012). It is for this reason they are commonly used as fecal indicator bacteria (FIB) to determine the microbiological quality of environmental waters. Most enterococci are commensal bacteria harmless to healthy humans, however, particular strains are opportunistic pathogens. These strains currently rank amongst the most prevalent multidrug resistant hospital pathogens worldwide (Gilmore *et al.* 2013), are the third most commonly isolated healthcare pathogen (Hidron *et al.* 2008), and are the leading aetiological cause of nosocomial infections (Kayser 2003).

The genus *Enterococcus* includes 36 species, most of which fall into five main groups: *E. faecalis*, *E. faecium*, *E. avium*, *E. gallinarum* and *E. cecorum* (Byappanahalli *et al.* 2012). Amongst these *E. faecalis* (80–90%) and *E. faecium* (less than 10%) are most commonly associated with humans and anthropogenic diseases (Duprè *et al.* 2003; Abriouel *et al.* 2008; Byappanahalli *et al.* 2012). Their pathogenic significance is enabled by a high frequency of genetic recombination and horizontal gene transfer. Of particular concern is the high prevalence of clinical *E. faecalis* and *E. faecium* isolates harbouring multiple antibiotic resistance genes either intrinsically or on mobile DNA elements

doi: 10.2166/wh.2017.284

(Byappanahalli *et al.* 2012; Gilmore *et al.* 2013). Typically, clinical *E. faecalis* strains harbour a higher number of virulence traits, whereas clinically derived *E. faecium* strains harbour the widest array of antibiotics (Byappanahalli *et al.* 2012). The increasing percentage of multi-drug resistant clinical strains also raises concern about the rate of genetic exchange between virulent and commensal strains outside hospital environments (Abriouel *et al.* 2008; Byappanahalli *et al.* 2012). This is supported by recent epidemiological studies that have positively correlated enterococci concentrations in coastal surface waters with increased incidences of swimmer gastroenteritis (Harwood *et al.* 2004; He & Jiang 2005; Grammenou *et al.* 2006). Consequently, enterococci have been used as FIB and are routinely used in water microbiology laboratories to determine the microbiological quality of recreational waters (WHO 2003; NHMRC 2008).

Whilst *E. faecalis* and *E. faecium* are most commonly associated with humans, enterococci are widely disseminated throughout many terrestrial and aquatic environments, both indirectly through wastewater treatment plants (WWTPs) and numerous agricultural activities, and directly through human contact (Byappanahalli *et al.* 2012; Varela *et al.* 2013). Although these environments exert various biotic and abiotic stresses on enterococci that inevitably lead to their decline, recent studies have found particular enterococci strains that can persist within these environments (Anderson *et al.* 2005; Signoretto *et al.* 2005; Ran *et al.* 2013). Furthermore, a number of studies have reported the persistence and distribution of enterococci and, in some cases, the presence of several virulence traits in strains isolated from coastal and marine surface waters (Abriouel *et al.* 2008; Badgley *et al.* 2010; Ran *et al.* 2013). In this study, we investigated the prevalence and persistence of enterococci populations and their virulence determinants in a large metropolitan river after an extreme flood event.

METHODS

Sampling sites and collection of water samples

On the 11 January 2011, the Brisbane River, a major metropolitan river in Southeast Queensland, Australia, broke its banks and many adjacent metropolitan areas were inundated with flood water. Between nine and 15 sites of the 23 selected sites along the river were sampled 4 (nine sites), 6 (15 sites) and 8 (12 sites) weeks after the major flood. The sampling sites and locations of nearby WWTPs relative to the sampling sites were previously described (Masters *et al.* 2017). A 1.5 L 'grab sample' was collected from each site in a sterile bottle from 30 cm below the water surface at the middle distance of the river, and transported on ice to the laboratory. Samples were processed within 12 h of collection from the first sampling site.

Enumeration, isolation and confirmation of *Enterococcus* spp.

The membrane filtration method was used to enumerate enterococci bacteria in water samples according to EPA Method 1600 (US EPA 2002). Triplicate serial dilutions of samples were filtered through 0.45 μm pore size nitrocellulose membranes (Millipore, Australia), and placed on mEI agar (Difco, Australia) plates and incubated at 41 ± 0.5 °C for 24 h. Enterococci were enumerated on plates with 30–300 colonies.

Up to 20 presumptive enterococci were then isolated from each sample, purified twice on m-*Enterococcus* agar (Difco) and stored at −80 °C until further testing. Presumptive identification of the isolates was carried out by testing for esculin hydrolysis using bile esculin agar (Oxoid, Australia) and incubated at 45 °C for 1 h to confirm their identification (Manero & Blanch 1999).

Total DNA extraction of water samples

The total enterococci DNA from 1 L samples was enriched by filtering water through a 0.45 μm pore size nitrocellulose membranes (Millipore). Filters were then placed on mEI agar (Difco) plates and incubated at 41 ± 0.5 °C for 24 h after which they were transferred to sterile flasks containing 50 mL of tryptic soy broth (Oxoid, Australia) and incubated at 37 °C overnight at 100 rpm on a rotary shaker. This enrichment step was included to ensure low numbers of *Enterococcus* spp. containing virulence genes (VGs) could be detected. DNA was extracted from 2 mL of the enriched broth using a DNeasy blood and tissue kit (Qiagen,

Australia) according to manufacturer's instructions and the resulting DNA extracts were stored at −20 °C until use.

Typing

All enterococci strains were typed using a high-resolution biochemical fingerprinting method using PhP-RE plates (PhPlate AB, Stockholm, Sweden) specifically developed for typing enterococci. The system measures the kinetics of bacterial growth in liquid medium of 11 different substrates in a microtiter plate (Kühn *et al.* 2003). Growth media and culture conditions were as previously described (Cassanovas-Massana & Blanch 2013). In brief, bacterial colonies were suspended in the first well of each row containing 325 μL of growth medium consisting of 0.011% w/v bromothymol blue, 0.02% w/v phosphate buffered saline (pH 7.4) and 1% w/v proteose peptone. Aliquots of 25 μL of bacterial suspensions were transferred into each of the remaining 11 wells containing 150 μL of growth medium. Plates were then incubated at 37 °C and scanned at intervals of 16, 40 and 64 h using a HP Scanjet 4890 flatbed scanner. After the final scan, the PhPlate software (PhPlate software v6.1) was used to create absorbance (A_{620}) data from the scanned plate images and the mean absorbance in each well over the three readings was calculated, yielding a biochemical fingerprint (BF) for each isolate. The BFs of the isolates were compared pair wise and the similarity among the isolates was calculated as correlation (similarity) coefficient and clustered according to the unweighted pair-group method with arithmetic averages to yield a dendrogram (Kühn *et al.* 2003). In the dendrogram, isolates with identical BFs were grouped into a common biochemical phenotype (C-BPT), whereas those having unique FPs were named as single (S) BPTs. The phenotypic diversity among the isolates was measured with Simpson's index of diversity (Di) (Ahmed & Katouli 2008). The similarity between different bacterial populations in two or more samples was calculated as a population similarity coefficient as previously described (Masters *et al.* 2017).

Polymerase chain reaction (PCR) detection of VGs

From each C-BPT, a representative isolate was selected and its genomic DNA was extracted using DNeasy blood and tissue kit (Qiagen) according to the manufacturer's instructions. The DNA extract of the representative isolates and the total DNA extract (TDE) of enterococci populations were screened for the presence of seven enterococci VGs. Detection of VGs was performed using previously published primers (Table 1). PCR reaction mixtures consisted of 5 μL of DNA, 20 pmol of each primer, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each of dNTP, 1.5 U Taq DNA Polymerase (Invitrogen Life Technologies, Australia) and sterile double distilled water (ddH_2O) to a total volume of 50 μL. PCR cycling conditions have been reported elsewhere (Shankar *et al.* 1999; Mannu *et al.* 2003; Vankerckhoven *et al.* 2004). PCR amplification was performed using an Eppendorf Master Cycler (Eppendorf, Australia). To detect the amplified product, up to 8 μL aliquots of the PCR product were visualised by electrophoresis at 130 V for 60 min using a 1.5% agarose gel (Progen, Australia) in 0.6× Tris/Borate/EDTA buffer pre-stained with ethidium bromide. Bands were visualised under ultraviolet light. Identification of the bands was established by comparison of the band sizes with molecular weight markers of a 100 bp ladder (GeneWorks, Australia). Samples were considered to be positive for a specific VG when the visible band was the same size as that of the positive control DNA. To identify false positive results ddH_2O was used as a template in the negative control in all experiments.

Biofilm formation

The same representative isolate of each C-BPT was also tested for biofilm formation by growing cultures overnight in Luria Bertani broth at 37 and 25 °C. The cells were pelleted, resuspended in fresh broth and adjusted to an absorbance of 1.00 at 600 nm, and subsequently diluted 1:100 in M9 minimal salt broth according to Schembri & Klemm (2000). Biofilm formation was tested at 25 and 37 °C. All test results were expressed according to criteria from Duprè *et al.* (2003).

Antibiotic resistance profiling

The representative isolates of each C-BPT were also tested for their resistance to 14 commonly used antimicrobial drugs according to the Clinical Laboratory Standard Institute

Table 1 | List of PCR primers and products used to detect enterococci VG

VG	Gene characteristic	Primer sequence (5′–3′)	Annealing temp. (°C)	Product size (bp)	Reference
AS	Aggregation substance	F: CCAGTAATCAGTCCAGAAACAACC R: TAGCTTTTTTCATTCTTGTGTTTGTT	54	406	Mannu *et al.* (2003)
ace	Collagen adhesin	F: AAAGTAGAATTAGATCCACAC R: TCTATCACATTCGGTTGCG	56	320	Mannu *et al.* (2003)
*gel*E	Gelatinase	F: AGTTCATGTCTATTTTCTTCAC R: CTTCATTATTTACACGTTTG	56	402	Mannu *et al.* (2003)
*efa*A	Cell wall adhesin	F: CGTGAGAAAGAAATGGAGGA R: CTACTAACACGTCACGAATG	56	499	Mannu *et al.* (2003)
esp	Cell surface protein	F: TTACCAAGATGGTTCTGTAGGCAC R: CCAAGTATACTTAGCATCTTTTGG	58	913	Shankar *et al.* (1999)
*cyl*A	Cytolysin	F: ACTCGGGGATTGATAGGC R: GCTGCTAAAGCTGCGCTT	56	688	Vankerckhoven *et al.* (2004)
hly	Hyaluronidase	F: ACAGAAGAGCTGCAGGAAATG R: GACTGACGTCCAAGTTTCCAA	56	276	Vankerckhoven *et al.* (2004)

(CLSI) method (CLSI 2011). The antimicrobial impregnated disks (Oxoid, Australia) tested were ampicillin (10 μg), vancomycin (30 μg), erythromycin (15 μg), tetracycline (30 μg), doxycycline (30 μg), ciprofloxacin (5 μg), norfloxacin (10 μg), chloramphenicol (30 μg), quinupristin-dalfopristin (15 μg), linezolid (30 μg), rifampin (5 μg), streptomycin (300 μg), gentamicin (10 μg) and nitrofurantoin (300 μg). The CLSI (2011) method records antimicrobial susceptibilities as either 'susceptible', 'intermediate' or 'resistant' and therefore the results were not expressed as MIC values.

PCR amplification of 16S rRNA and sequencing

Representatives of each C-BPT were 16S rRNA sequenced using the universal primers specific for Bacteria and Achaea (Manero & Blanch 2002). Each reaction consisted of 1 × Mango mix (Bioline, Australia), 10 μM primers (27F 5′-AGAGTTTGATCCTGGCTCAG-3′ and 1492R 5′-GGTTACCTTGTTACGACTT-3′), 1 μL of DNA template plus ddH_2O to reach a final reaction volume of 25 μL. The 16S rRNA fragment was amplified in an Eppendorf Master Gradient Thermal Cycler (Eppendorf) with the following program: initial denaturation 95 °C for 3 min, 30 cycles of 30 s at 94 °C, 30 s at 50 °C and 3 min at 72 °C, and a final extension at 72 °C at 6 min. The PCR product was then purified using a Qiaquick PCR purification kit (Qiagen), according to the manufacturer's instructions. Bidirectional sequences were obtained using 27F and 1492R primer targeting sites on either side of the insert. DNA sequencing was subsequently carried out at the Australian Genome Research Facility Ltd (St Lucia, Queensland, Australia). The raw gene sequences were processed using the Sequencher software (Gene Codes, Ann Arbor, MI, USA). For the 16S rRNA gene sequences, homology searches of DNA sequences in the GenBank (non-redundant) database were undertaken with the National Center for Biotechnology Information (NCBI) BLAST program (www.ncbi.nlm.nih.gov/BLAST/) (Altschul *et al.* 1997).

Statistical analysis

A one way analysis of variance (ANOVA) test was used to compare the significance of difference between compared groups and sampling rounds. Linear regression analysis was applied to investigate the degree of correlation between the number of enterococci and (1) the number of BPTs at each site, (2) the Di of isolates and (3) the VGs observed at each site. Linear regression analysis was also applied to the number of VGs possessed by each C-BPT and the number of antibiotics to which it was resistant.

RESULTS

Enumeration of enterococci

The highest concentration of enterococci was observed at site 9 during the first round of sampling, exceeding 59,000 colony forming units (CFU)/100 mL (Table 2). This site was adjacent to a major storm water outlet and downstream of a WWTP (approximately 4 km) (Figure 1). This was significantly higher ($P < 0.001$) than the enterococci concentrations detected across all other samples. The mean number of enterococci found across round one (except site 9) sampling was not significantly higher than that of round two, however, the mean number of these bacteria in samples from round three were significantly ($P < 0.05$) lower than that of round two (Table 2).

Table 2 | Enumeration of enterococci (CFU/100 mL) found at each sampling site and across three sampling rounds

Site code	Sampling round 1 Mean ± SD	Sampling round 2[b] Mean ± SD	Sampling round 3[b] Mean ± SD
1	NT	20 ± 8	NT
2	NT	143 ± 5	74 ± 98
3	NT	NT	47 ± 12
4	NT	210 ± 24	NT
5	NT	NT	30 ± 10
6	557 ± 48[a]	343 ± 42	60 ± 26
7	NT	420 ± 73	NT
8	829 ± 104[a]	NT	117 ± 23
9	59,800 ± 7,550[a]	NT	187 ± 15
10	555 ± 48[a]	NT	NT
11	765 ± 107[a]	NT	217 ± 76
12	NT	2,363 ± 427[a]	NT
13	903 ± 138[a]	NT	357 ± 55
14	2,800 ± 1,006[a]	NT	707 ± 330[a]
15	NT	3,290 ± 316[a]	NT
16	407 ± 48	2,530 ± 235[a]	200 ± 53
17	394 ± 62	943 ± 145[a]	190 ± 35
18	NT	1,593 ± 115[a]	330 ± 58
19	NT	1,213 ± 73[a]	NT
20	NT	1,063 ± 172[a]	NT
21	NT	777 ± 135[a]	NT
22	NT	630 ± 33[a]	NT
23	NT	513 ± 61[a]	NT
Mean ± SD	901 ± 790[c]	1,070 ± 974	210 ± 189

NT indicates sites that were not tested at each sampling round.
[a]Enterococci concentrations above Category D of Australian recreational water quality guidelines (NHMRC 2008).
[b]Significant difference ($P < 0.05$).
[c]Mean value excluding the site 9 value.

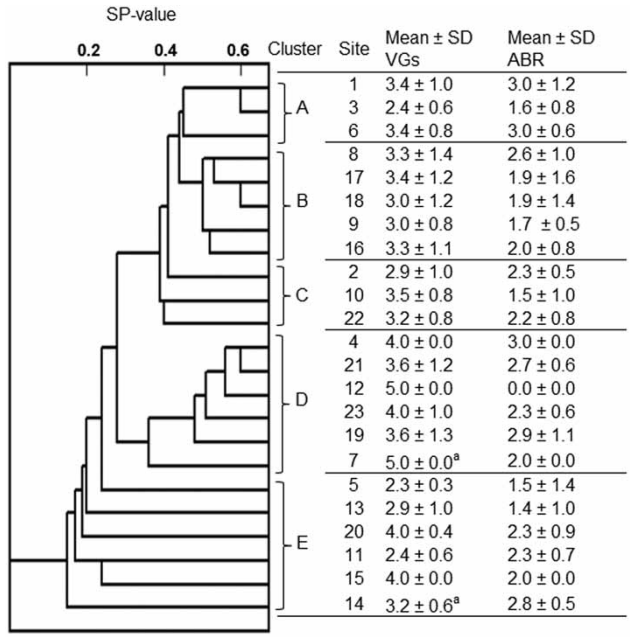

Figure 1 | Similarity among the populations of enterococci (C-BPTs) at the different sites, measured as a population similarity coefficient (SP-value). The mean number of VGs and antibiotic resistance (ABR) is indicated for each sample site. [a]Indicates those clusters that have a significantly ($P < 0.05$) higher number of C-BPT detected at those sites.

Typing

A total of 482 isolates was confirmed as enterococci. Typing of these isolates yielded 23 common C-BPTs comprising of 350 isolates (72.6%) and 132 single S-BPTs. Among the C-BPTs, all but two were found at more than one sampling site (Figure 2). Ten C-BPTs (representing >59% of all isolates) were found in more than one sampling round, with one C-BPT (i.e. C23) found at all sample rounds and represented 19.9% of the isolates tested.

Similarity among the populations of enterococci at different sites was measured as an Sp coefficient. Sites belonging to clusters A to B were found to be highly similar with an Sp > 0.4 (Figure 1). Cluster A contained sites closer to the mouth of the river whereas cluster B contained sites

C-BPT	Percent of total	Sampling Sites																						Virulence genes								VG (/7)	Biofilm (25°C)	Biofilm (37°C)	Antibiotic resistance												*Enterococcus* spp.
		1[b]	2[b,c]	3[c]	4[b]	5[c]	6[a,b,c]	7[b]	8[a,c]	9[a,c]	10[a]	11[a,c]	12[b]	13[a,c]	14[a,c]	15[b]	16[a,b,c]	17[a,b,c]	18[b,c]	19[b]	20[b]	21[b]	22[b]	23[b]	*cylA*	*hyl*	*esp*	*ace*	*gel*	*efaA*	AS				C30	RD5	CIP5	F300	NOR10	E30	QD15	DO30	TE30	AMP10	Total	
C1	0.8														a		a								●		●	●	●		4				●	○				●				3	*E. faecalis*
C2	0.8									a					a											●	●	●	●		4				●	●		○		●				4	*E. faecalis*
C3	0.8									a		a		a											●				●		2													0	*E. mundtii*
C4	1.8									a	a						a	a							●			●	●		3							○						1	*E. casseliflavus*
C5	3.0						a			a	a			a				a							●	●	●		●	●		5	X	X											0	*E. faecium*
C6	0.6												b								b			b	●	●			●	●		4					○		○						2	*E. casseliflavus*
C7	1.2															b												●	●		2	X												0	*E. mundtii*
C8	0.6																	c	c										●	●		2													0	*E. casseliflavius*
C9	0.6								c										c						●			●	●	●		4				●	○	○	○						4	*E. faecium*
C10	0.6		c											c															●	●		2							○						1	*E. casseliflavius*
C11	0.8		c																	c		c					●		●	●		3				●		●		○					3	*E. faecium*
C12	1.0		c									c			c															●		1	XX			●				○				●	3	*E. faecium*
C13	1.2			c		c													c											●		1				●									1	*E. faecalis*
C14	1.2							b							a	b		a		b	b				●	●	●		●	●		5				●					●				2	*E. gallinarium*
C15	1.8									a				a	a		b												●	●		2	X								●				1	*E. mundtii*
C16	1.8	b								a		a			a			b		b						●				●		2			●	○	●	●	●						5	*E. hirae*
C17	7.5	b	b		b		a		a	a	a	a		a			b			b	b	b	b	b		●				●	●	3				●	○		●						3	*E. casseliflavus*
C18	3.5		c			c			c					ac	a			a							●		●		●	●		4				●	○		●						3	*E. faecalis*
C19	4.7	b	b	c			c								c	b			c	b	b					●				●	●		3					○		○						2	*E. mundtii*
C20	2.2		b				a		a								c	abc	c				b			●	●			●	●		4				●	○		●						3	*E. casseliflavus*
C21	5.5	b	b	c			abc		a	c	a	c		c	c		bc	b	c		b		b			●				●	●		3				●	○		●						3	*E. casseliflavus*
C22	10.9		bc	c		c	a		c	c	a	c		c			bc	abc	bc				b								●		1													0	*E. mundtii*
C23	19.9	b	bc	c	b	c	abc	b	ac	ac	a	ac	b	ac	ac	b	abc	ac	bc	b	b	b	b	b	●	●			●	●		4	XX									●	●		2	*E. casseliflavus*

Figure 2 | C-BPTs of enterococci found at sampling sites along the Brisbane River over three rounds, e.g. 'ab' indicates C-BPTs found during round 1 and 2. A representative isolate from each C-BPT was screened for resistance to 14 antimicrobial agents (● indicates resistance and ○ indicates intermediate resistance), 7 enterococci VGs, biofilm formation (X indicates weak biofilm production, XX indicates moderate biofilm production). Species identification was determined by sequencing 16S rRNA. Antibiotics screened included C = chloramphenical, RD = rifampin, CIP = ciprofloxacin, F = nitrofurantoin, NOR = nofloxacin, E = erythromycin, QD = quinupristin-dalfopristin, DO = doxycycline, TE = tetracycline, AMP = ampicillin (numbers indicate concentration in μg).

within the central business district and high density residential area of the catchment.

Prevalence of enterococci VGs

All C-BPTs harboured at least one VG with C5 (found only in round 1) and C14 (found in rounds 1 and 2) harbouring five VGs (Figure 2). The most prevalent VGs were *efa*A and *gel*, which were found in 23 (100%) and 17 (74%) of C-BPTs, respectively. The VGs *cyl*A, *hyl*, *esp* and *ace* were found in isolates representing multiple C-BPTs and were detected in 10 (44%), nine (39%), five (22%) and three (13%) of these types, respectively. Whilst the VG AS was only detected in one C-BPT (C17), this C-BPT represented 7.5% of all enterococci tested (Figure 2). No significant difference was detected in the number of VGs belonging to each of the C-BPT across the three sampling rounds (Figure 2).

Biofilm formation was exhibited in five C-BPTs at 25 °C and in one C-BPT at 37 °C (Figure 2). Resistance (including intermediate) was observed in all but five of the 23 C-BPTs. Fourteen C-BPTs were multidrug resistant with the highest resistance (i.e. resistant to five ABs) observed in one C-BPT (i.e. C16) (Figure 2). Resistance to rifampin and the two fluoroquinolones, i.e. norfloxacin and ciprofloxacin, was most common amongst C-BPT populations; 12 (52%), 11 (48%) and 10 (43%), C-BPTs, respectively. Resistance to streptogramin, quinupristin-dalfopristin, nitrofurantoin and erythromycin was found in four (17%), three (13%) and two (9%) of the C-BPTs, respectively. None of the C-BPTs exhibited resistance to vancomycin; therefore no genotypic evaluation for vancomycin genes was conducted. Eight of the 10 C-BPTs found at more than one sampling round, representing 222 enterococci isolates (46%), were multidrug resistant (Figure 2). Strains belonging to C-23 representing 96 isolates (20%) harboured four VGs, exhibited strong biofilm formation at 25 °C and were resistant to two antibiotics (Figure 2).

Species identification

16S rRNA sequencing revealed six species of *Enterococcus* (Figure 2). The most common species, i.e. *E. casseliflavus*

Table 3 | Number of VGs found in TDE of enterococci and the number of BPTs found in each sample

Sampling round	Site	No. C-BPTs	No. S-BPTs	No. VGs in total enterococci DNA	Di
1	6	5	1	6	0.89
	8	6	2	5	0.91
	9	9	4	5	0.88
	10	6	2	4	0.92
	11	7	4	4	0.89
	13	9	2	4	0.94
	14	10	2	5	0.91
	16	8	5	6	0.96
	17	10	6	6	0.85
Mean ± SD		7.8 ± 1.9[a]	3.1 ± 1.7	5.0 ± 0.87	0.91 ± 0.03
2	1	5	3	5	0.92
	2	10	6	6	0.94
	4	1	2	5	0.70
	6	5	2	5	0.86
	7	1	1	5	0.46
	12	1	1	5	0.56
	15	1	4	5	0.94
	16	8	4	6	0.77
	17	10	4	6	0.88
	18	8	5	5	0.80
	19	6	0	6	0.83
	20	6	5	6	0.99
	21	2	4	5	0.77
	22	4	4	5	0.96
	23	3	1	5	0.73
Mean ± SD		4.7 ± 3.2[a,b]	3.1 ± 1.8[b]	5.3 ± 0.49	0.81 ± 0.15
3	2	10	1	6	0.91
	3	5	7	5	0.96
	5	4	5	5	0.96
	6	5	4	5	0.83
	8	6	5	6	0.89
	9	9	3	7	0.86
	11	7	6	5	0.88
	13	9	6	5	0.96
	14	10	8	6	0.96
	16	8	4	6	0.83
	17	10	4	5	0.85
	18	8	5	5	0.80
Mean ± SD		7.6 ± 2.2[b]	4.8 1.9[b]	5.5 ± 0.67	0.89 ± 0.06

Di, Diversity index.
[a,b]Indicates a significant difference ($P < 0.05$).

represented 38.7% of all isolates, belonged to eight C-BPTs and were found in all water samples. The remaining species included *E. mundtii* (19.4%), *E. faecalis* (6.3%), *E. faecium* (5.4%), *E. gallinarium* (1.2%) and *E. hirae* (1.8%).

VGs in TDE of enterococci populations

The number of VGs detected in the TDE of enterococci did not differ significantly between the sites and the three sampling rounds (Table 3). A linear regression analysis applied (data not shown) found that the number of enterococci in the water samples did not correlate with the number of positive VGs observations ($P = 0.6306$).

There was, however, a significant ($P < 0.05$) decrease in the number of C-BPTs and S-BPTs between the sampling rounds (Table 3). High diversity was observed amongst the enterococci isolates in all sampling sites and rounds with no significant difference between the rounds (Table 3).

DISCUSSION

The persistence and prevalence of enterococci in recreational waters pose a serious risk to public health (Di Cesare *et al.* 2013). Typically, these health risks are much greater during storm events, where concentrations can spike by several orders of magnitude. The potential for the spread of their virulence factors to commensal populations can also increase significantly. The close interactions required for horizontal gene transfer in related but ecologically distinct bacterial strains, such as those belonging to the genus *Enterococcus*, are potentiated by the turbulent influx that mixes contaminants and resuspends particulate matter throughout the water column (Tendolkar *et al.* 2003).

In spite of the serious public health risks associated with exposure to contaminated waters (Byappanahalli *et al.* 2012) and the clinical significance of opportunistic enterococci (Fisher & Phillips 2009), there remains a paucity of knowledge on the distribution and persistence of *Enterococcus* spp. populations and their virulence characteristics within highly urbanised river systems after a significant flood event. In this study we surveyed enterococci along a large

metropolitan river, typical of most highly urbanised cities built in the low lying areas adjacent to a large water system.

As expected after such an extreme flood event, enterococci persisted well above Category D (>500 CFU.100 mL^{-1}) of the national recreational water guidelines (NHMRC 2008) for at least 6 weeks post-flood. National guidelines cite significant health risks associated with whole-body exposure to waters above this threshold, and predict >10% risk of GI illness (NHMRC 2008). The persistence of elevated enterococci concentrations found across most sites upstream of the tidal zone may have, at least in part, been attributable to the influx of untreated effluent entering the river for up to 8 weeks post-flood. This was the consequence of the extensive flood damage to the infrastructure of all but one WWTPs located within the catchment. Although we could not type enterococci from those WWTPs located within the catchment to confirm our speculation, we did find several *E. faecalis* and *E. faecium* populations persisting across all three sampling rounds of the study, and this correlates with the media reports at the time that untreated effluent entered the river throughout the duration of this study. Since these species are dominant constituents of the gut microbiota of humans and animals, their dominance in fecal-contaminated waters would be expected, as reported in various studies (Ferguson *et al.* 2005; Signoretto *et al.* 2005; Byappanahalli *et al.* 2012; Ryu *et al.* 2013).

To evaluate the population dynamics of enterococci in the river, we typed all confirmed enterococci isolates using the highly discriminatory PhP typing system. This method offers key advantages analogous to the scope of this study, and has been extensively reported in the literature as a reputable typing method with the discriminatory power comparable to genotypic typing methods (Kühn *et al.* 2003; Ahmed *et al.* 2006; Blanch *et al.* 2006; Anastasi *et al.* 2012; Cassanovas-Massana & Blanch 2013). Although it was not feasible to evaluate all sites over the three rounds, the distribution of the clonal groups detected in this study indicated several prevalent and persistent populations within the river system. 16S rRNA sequencing revealed that the most dominant populations were *E. casseliflavus*, which correlates with previous enterococci population studies conducted in coastal and/or riverine water systems in the United States, and Puerto Rico (Moore *et al.* 2008; Hernandez 2011; Ran *et al.* 2013). Moreover, the presence and relative abundance of the other *Enterococcus* spp. detected in this study was consistent with previous ecological studies (Ferguson *et al.* 2005, 2013; Badgley *et al.* 2010; Ran *et al.* 2013; Ryu *et al.* 2013) that identified these species (i.e. *E. mundtii*, *E. faecalis*, *E. faecium*, *E. gallinarium* and *E. hirae*) in aquatic and terrestrial vegetation, sand, sediment, and soil samples belonging to freshwater or beach environments. Although the majority of enterococci screened in this study belonged to a relatively small number of clonal groups, we reported very high population diversities across all sites and sampling rounds, which are in agreement with earlier studies (Ahmed *et al.* 2006; Bonjoch *et al.* 2011). In spite of the significant decrease in enterococci concentrations, the diversity in the samples remained comparably high across all sites and rounds. This suggests enterococci species of diverse source origins can persist and possibly survive within these secondary sources, as reported elsewhere (Anderson *et al.* 2005; Haller *et al.* 2009).

Irrespective of niche habitats, it would be expected that enterococci strains of fecal and WWTP origin harbour more virulence factors than those endemic to the environment as previously reported (Fisher & Phillips 2009), but our results do not support this. The strains most likely to be of clinical importance did not harbour the greatest number of VGs (i.e. *E. faecalis*) nor were they found to harbour the greatest resistance to antimicrobial agents (i.e. *E. faecium*). As highlighted by the presence of the *esp* gene, *E. faecium* is shown to be strongly associated with humans (Scott *et al.* 2005; Ahmed & Katouli 2008), yet this VG was found in other *Enterococcus* spp. which indicates cross-reactivity with other non-human sources as found by others (Byappanahalli *et al.* 2012; Di Cesare *et al.* 2013). The presence of multi-drug resistant strains in our enterococci populations raises further concerns of the spread of these virulent factors in aquatic reservoirs. We also found the presence of quinupristin-dalfopristin and chloramphenicol resistant strains in our samples. These antibiotics are commonly found in animals, soil and other non-human sources, and are normally used to treat multi-drug resistant and vancomycin resistant clinical enterococci, respectively (Arias *et al.* 2010). The clinical significance of these virulence determinants has been reported for those species typically associated with disease, however their role in commensal species of very different origin has not been reported. Important to this would be how these strains acquire their virulence determinants,

and whether these factors persist within the naturalised populations either to provide survival advantages in an aquatic environment or act as a sink for future dissemination. A preliminary study recently surveying antibiotic resistance in *E. faecalis* and *E. faecium* derived from marine sediment suggests efflux of antibiotic resistance may in fact occur unilaterally between environmental and clinical strains (Di Cesare *et al.* 2013). The presence of a dominant and persisting clone of enterococci in all sampling rounds of this study is an example of such strains. This clone was not only resistant to several antibiotics and carried a number of VGs, but also formed biofilm. Biofilm formation can be induced in stressed environments, and is suggested to aid in the persistence and survival of bacteria (Marshall *et al.* 2012). The biofilm production in this study was assessed at two different temperatures, and the fact that this persistent clone produced biofilm at temperatures closer to the water temperature, i.e. 25 °C, supports the role of this virulence factor in survival of this clone in the water.

CONCLUSIONS

This study demonstrated the presence and persistence of a few clones of enterococci within a major metropolitan river after an extreme flood event which inundated several WWTPs within the catchment resulting in releasing untreated sewage into the river. The most dominant and persistent clone carried a number of virulence factors including the ability to form biofilm which could aid survival and persistence of these bacteria in the river after flood. Exacerbated by increasing anthropogenic and climatic pressures; the ubiquity, genomic plasticity and increasing number of species associated with enterococcal illnesses raises serious public health concerns. We suggest that the diversity and persistence of enterococci populations and the wide dissemination of multiple virulence factors across a major water system highlight the potential of continued health risks in this catchment.

ACKNOWLEDGEMENTS

We would like to thank Jacob Gruythysen, John Ferris, Mark Davidson, Joel Ferris, Joel Hodge, Myles Waller, James Fels, Darren Renouf, Paul Hough and Rae Huggins from the Department of Science, Information Technology, Innovation and the Arts (DSITIA) for collecting the samples.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest with the organisation that sponsored this research and publications arising from this research.

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First received 21 October 2015; accepted in revised form 1 May 2017. Available online 14 June 2017